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(54) Title: A CHITOSAN POLYMER HAVING A SPECIFIC DEGREE OF ACETYLATION

(57) Abstract

The present invention relates to a pharmaceutical formulation comprising a chitosan polymer having a degree of acetylation of between 20 % and 45 % and a molecular weight of above 75,000, and a therapeutic active agent, a chitosan polymer for preparing said formulation, and the use of said chitosan polymer and a method of enhancing the absorption of a therapeutic active agent.

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A chitosan polymer having a specific degree of acetylation.

Field of the invention

The present invention relates to a new pharmaceutical formulation comprising a chitosan polymer having a specific degree of acetylation and a specific molecular weight, the chitosan polymer per se, and the use of the chitosan polymer.

Background of the invention

Chitin and its derivatives have long been known to have numerous potential beneficial uses, such as for example food ingredient or water purification aid (Chitin and chitosan, Eds.: G. Skjak-Braek, T. Anthonsen and P. Sandford, Elsevier, Amsterdam, 1989). Chitin is found abundantly in nature in for instance crab and shrimp shells, insects, and mushrooms. The primary units in chitin are 2-deoxy-2-acetylamino glucose units which are combined by (1,4) glycosidic linkages into a linear polymer. N-deacetylation of chitin results in chitosan, a high molecular weight cationic polysaccharide. The degree of N-acetylation is an important characteristic of chitosan, as it determines the positive charge of the molecule and its polarity. Chitosan may be manufactured with different degrees of acetylation as well as molecular weight (Anthonsen et al., Carbohydrate Polymers, 22, 1993, pp 193-201).

Chitosans are considered biocompatible macromolecules due to assumed low toxicity and biodegradability. They are degraded by lysozymes and related enzymes, such as

N-acetyl-D-glucosaminidases. The lysozymic digestibility of the polymer increases with the degree of N-acetylation (Aiba, Int. J. Biol. Macromol., 14, 1992, pp 225-228; Bourbouze et al., Clin. Chim. Acta, 199, 1991, pp 185-194). Their biocompatibility has initiated evaluation of the compound for many medical applications, e.g. in JP 0509295 A; Nishimura et al., Mol. Biother., 2, 1990, pp 115-120; Segal et al., Mycopathologia, 102, 1988, pp 157-163; Nishimura et al., Vaccine, 2, 1984, pp 93-99; Lida et al,. Vaccine, 5, 1987, pp 270-274; and JP 5414809 A. They are also useful as sustained or controlled release carriers for drugs as is disclosed e.g. in JP 04264023 A and EP 460921-A.

In the patent US 4873 092 by Azuma et al chitosans are used as carriers for physiologically active substances, such as adriamycin and testosterone, with the aim to slowly release these substances from the carrier and therewith obtain constant therapeutic (but non-toxic) blood concentration of the drug over an extended period of time. Chitosans that could be used as such slow release carriers are stated to have a degree of N-acetylation of 0-100% and a molecular weight of 10000 to 23000. A paper by Imai et al, Int. J. Pharm., 67, 1991, pp 11-20, describes the use of chitosans with low molecular weight (<25.000) and intermediate degrees of acetylation (10-34%) as carriers for the oral absorption of indomethacin. These chitosans increases the in vitro dissolution of the acidic drug. Since dissolution of the poorly water soluble indomethacin is rate limiting for its oral absorption, the increased dissolution results in an increased oral absorption of the drug.

Chitosans have been found to have mucoadhesive properties, and due to their close contact with the

mucosal epithelium may prove useful in delivery of drugs to mucosal absorption sites such as the small intestine or colon and rectal mucosa (EP 514008-A1; Lehr et al., Int. J. Pharm., 78, 1992, pp 43-48).

For various hydrophilic drugs such as several antibiotics, peptides and proteins the solubility of the drugs is not rate limiting in the absorption process. Many of these compounds, however, are poorly absorbed following oral administration due to extensive enzymatic degradation and poor epithelial permeability. In order to overcome the physical barriers that hinder drug absorption it is often inevitable to coadminister so called absorption enhancers. Absorption enhancers as used in the present patent application refer to compounds that increase drug absorption by an interaction between the mentioned compound and the mucosal epithelium. They do not include compounds that increase drug absorption by an interaction with the drug itself. Numerous classes of compounds have been described that improve the intestinal absorption of hydrophilic and high molecular weight compounds. Such absorption enhancing compounds include surfactants, bile salts, chelating agents, and fatty acids. (Lee et al., Crit. Rev. Therapeut. Drug Carrier Syst., 8, 1991, pp 91-192). A major concern, however, related to the use of many of such absorption enhancing agents is potential adverse effects on epithelial integrity, morphology, and function (Swenson and Curatolo, Adv. Drug Delivery Rev., 8, 1992, pp 39-92). Moreover, the delivery of an effective amount of enhancer and drug at the site of absorption to get reproducable dosing is necessary, but difficult to achieve. In addition, the onset of action of the enhancer and the duration of its activity should coincide with the presence of the drug at the absorption site and the time necessary for its uptake (De Boer and Breimer, In Drug absorption

enhancement, Eds.: De Boer, Harwood Ac. Publ., Chur, 1994, pp 155-175). Compounds with optimal absorption enhancement characteristics, thus should have a strong effect on epithelial permeability, an early onset of action, and a low toxicity.

Recently, chitosans have been investigated as potential absorption enhancing compounds to aid in the absorption of hydrophilic, and high molecular weight drugs across mucosal tissues. Chitosans have been found to enhance the mucosal absorption of insulin, a small model peptide, and a hydrophilic model marker molecule. In a published patent application by Illum et al. (WO 9009780-A), it is described that the chitosans Seacure and Seacure+ improve the absorption of insulin across the nasal mucosa of sheep and rat to a large extent. The concentration of the chitosan used in these studies is 0.1 to 0.5% (w/v). Seacure+ is stated to be a water soluble chitosan glutamate with either a low (Seacure+) or an intermediate viscosity (Seacure+210). The degree of acetylation or the molecular weight of the chitosan are not clearly stated. Effective polymers, however, are stated to be chitosans in a molecular weight range of 10,000 to 500,000.

The same water soluble chitosan (Seacure+ 210) has been used to study chitosan effects on Caco-2 cell monolayers (Artursson et al., Pharm. Res., 1994, 11, pp 1358-1361). Caco-2 is a human intestinal epithelial cell line that is commonly used as a model for human intestinal epithelium. The Seacure+ chitosan increases the permeability of the epithelium 5 to 10 fold as measured by the transport of a model hydrophilic compound across the epithelium. Seacure+ is used at concentration from 0.1 to 0.5% (w/v) and the pH of the solutions ranges from 4.0 to 6.0.

Intermediate viscosity Seacure +210 has in another study (Rentel et al., Proc. Intern. Symp. Control. Rel. Bioact. Mater., 20, 1993, pp 446-447) been found to improve the transport of a small peptide across intestinal tissue in vitro. In the same study a different chitosan was also tested. This chitosan, Daichitosan VH (DA=15-20%; Mw=500000-800000) showed similar improvement of transport of the small peptide across intestinal mucosa at a concentration of 1% (w/v).

Although all the presented studies show improvement of mucosal epithelial permeability in the nose as well as in the intestinal tract in several animal species, including sheep and rat as effectuated by chitosans at different concentrations (0.1-2% (w/v)) and at different pH values, they do not elucidate the influence of the two important characteristics of chitosans on the absorption enhancing effect, namely the degree of acetylation and the molecular weight. In fact in the described papers none of the parameters are very well specified.

Brief description of the invention

According to the present invention it has unexpectedly been found that intermediate but not high degrees of acetylation, i.e. a degree of acetylation of between 20 and 45%, and a high molecular weight, i.e. a molecular weight of above 75,000, of chitosans are necessary for increasing epithelial permeability, to achieve an early onset of absorption enhancement, and a low toxicity of the enhancer.

Detailed description of the invention

Studying the transport of the marker molecule, mannitol, as a model for hydrophilic drugs (Anderberg et al., Pharm. Res., 10, 1993, pp 857-864), and the

cardiovascular drug, atenolol, across Caco-2 cell monolayers it was found that chitosans with a low to intermediate degree of acetylation and/or a large molecular weight are necessary in order to enhance permeability. That is, chitosans with a degree of acetylation of 1% (DA=1%) and either a small molecular weight (Mw=31000) or a large molecular weight (Mw=170000) have a strong concentration dependent effect on mannitol transport. At concentrations as low as 50 µg/ml (0.005% (w/v)) they both increase mannitol transport 10- to 15-fold. At a concentration of 250 µg/ml the increase in permeability is 50-fold. Chitosans with an intermediate (DA=35%) or high degree of acetylation (DA=49%) and a large molecular weight (Mw=170000 and 98000, respectively) also improve mannitol transport. Concentrations of 50 and 250 µg/ml, respectively (0.005 and 0.025% (w/v)) are necessary to achieve a 10 to 15 fold increase in mannitol permeability of the Caco-2 cell monolayers. The increase in mannitol permeability of the latter chitosans does not have such a steep concentration/effect relation as is observed for the former chitosans with a low degree of acetylation (DA=1%). On the other hand, chitosans with an intermediate or a high degree of acetylation and a small molecular weight (chitosan (DA=35%, Mw=5300), chitsoan (DA=35%, Mw=12000), chitosan (DA=49%, Mw=22000)) do not have an effect on mannitol transport at all. Concentrations upto 250 µg/ml (0.025% (w/v)) do not change the permeability of mannitol across Caco-2 cell monolayers significantly.

Although at a concentration of 50 µg/ml chitosans with a degree of acetylation of 1% and a molecular weight of 31000 and 170000, and chitosans with a degree of acetylation of 35% and a molecular weight of 170000, and at a concentration of 250 µg/ml chitosan with a

degree of acetylation of 49% and Mw of 98000 all show similar enhancement of mannitol permeability across Caco-2 cell monolayers, their adverse effects on epithelial cells are surprisingly very different. In a toxicity assay which measures intracellular dehydrogenase activity and is based on the observation that injured cells display a reduced dehydrogenase metabolism, a much stronger toxicity was observed for chitosans with a low degree of acetylation (DA=1%, Mw=31000 or 170000), than for chitosans with an intermediate and high degree of acetylation and a large molecular weight (chitosan (DA=35%, Mw=170000), chitosan (DA=49%, Mw=98000). These findings were confirmed by visualisation of the adverse effects of chitosans on epithelial Caco-2 cell monolayers with transmission electron microscopy, which showed that the morphological changes following exposure to chitosans with intermediate and high degree of acetylation and high molecular weight (chitosan DA=35%, Mw=170000; chitosan DA=49%, Mw=98000) were small, while clear changes were observed following exposure to chitosan with a degree of acetylation of only 1%. These changes involved dicontinuities and reduced number of microvilli, and a disorganized terminal web.

In addition, the onset of action of increased epithelial permeability is much quicker for a chitosan with intermediate degree of acetylation (35%) and large molecular weight (170000), than for chitosan with a low degree of acetylation and small or large molecular weight (DA=1%, Mw=31000 or 170000). This gives the former chitosan more controllable and reliable absorption enhancing characteristics.

In contrast to the findings of Imai et al. (Int. J. Pharm., 67, 1991, pp 11-20) who found that chitosans with low molecular weight were necessary to increase

the dissolution of acidic drugs, we have found that, in order to improve drug absorption by means of increased epithelial permeability for hydrophilic drugs, chitosans with high molecular weight and intermediate degree of acetylation are preferable with regard to improved permeability, early onset of action, and low toxicity.

The invention provides a pharmaceutical formulation of a form and composition suitable for mucosal administration, particularly for oral and/or rectal delivery, of therapeutic agents in man. By oral route means e.g. buccal, intragastric and intestinal. This preparation comprises a therapeutic active agent as well as a chitosan with the below described characteristics as an absorption enhancing agent.

The present invention is based on the observation that it is possible to select chitosans with optimal absorption enhancement characteristics, that is chitosans with a strong effect on eptithelial permeability, low toxicity, and an early onset of action. Such a chitosan would, according to the present invention have a degree of acetylation of between 20 and 45%, and preferably between 30 and 40%, while its molecular weight should be above 75,000, more preferably between about 75,000 and 250,000, and most preferably between about 98,000 and 200,000.

Therapeutic active agents include drugs, vaccines, proteins, peptides and fragments thereof. The pharmaceutical formulation according to the invention may be used with drugs from the following non-exlusive list.

Pharmaceutical formulations containing hydrophilic drugs with low membrane permeability and biovailability

which are absorbed mainly via the paracellular route such as;

- Blood pressure lowering agent such as hydrophilic ACE-inhibitors and β-blockers

- Anti-coagulation agents such as thrombin inhibitors heparin and low molecular weight heparin

- Antidiuretic drugs and vasopressin analogs such as DDAVP, AVP, etc.

- Peptide hormones such as insulin, calcitonin, growth hormone, PTH

- Antibiotics such as penicillin and penicillin derivatives, tetracycline, and macrolides

- Steroidal anti-inflammatory agents such as beclomethasome and budesonide

- Non-steriodal anti-inflammatory agents such as diclofenac, indomethacin, ibuprofen, naproxen

- Coagulation factors such as Factor VIII

- Vaccines and antigens such as polio vaccine

- Anti-viral drugs such as Foscarnet

- Compounds effecting bone metabolism such as clodronate and other bisfosfonates

- Analgesics such as opioid peptides, dextropropoxifene and pentazocine

- Local anesthetics with antiinflammatory properties

such as lidocaine, bupivacaine and ropivacaine

- Local anesthetics with analgetic properties such as bupivacaine and ropivacaine

- Immunomodifiers such as Cyclosporin A

- Anti-allergic drugs such as sodium cromoglycate

The pharmaceutical formulations according to the invention will in practice contain a pharmacologically active amount of therapeutic agent. As regards the amount of chitosan the formulation will contain an amount thereof that enhances the absorption of the active agent present. Hereto, it will be necessary to scale the dose of absorption enhancer from in-vitro cell culture studies and animal experiments to the therapeutic situation in man. Such a dose scaling will be common for anyone skilled in the art. Chitosan amounts of 5 µg/dose to 1000 mg/dose are possible. Amounts of 50 µg to 500 mg/dose are preferable.

If desired, other absorption enhancers may be included in the pharmaceutical formulations of the invention. Such enhancers can be inhibitors of enzymatic degration, in order to reduce the amount of drug that is metabolized before absorption. Enzyme inhibitors used to stabilize drugs in mucosal epihelia and the gastrointestinal lumen include bile salts, bacitracin, aminoboronic acid derivatives, bestatin, FK-448, soybean trypsin inhibitor, and aprotin (Muranishi and Yamamoto, In Drug absorption enhancement, Eds.: De Boer, Harwood Ac. Publ., Chur, 1994, pp.67-100).

Formulations suitable for the delivery of the drugs to the small intestine or colon can utilize coated solid dosage forms or enteric systems, which will loose their

coating on arrival in the small intestine due to dissolution of the coating polymers at the local pH conditions. Colon specific delivery systems may involve polymer coatings that typically are degraded by the colonic bacterial flora. Upon degradation of the coating these delivery systems will release drug and additives.Upon degradation of the coating, these delivery systems will release drug and additive (Friend, Advanced Drug Delivery Reviews, 7, 1991, pp 149-201) Such polymer coatings may for instance comprise azopolymers (Saffran et al., Science, 233, 1986, pp 1081-1084), and polysaccharides such as calcium pectinate (Rubinstein, Pharm. Res., 10, 1993, pp 258-265).

Alternatively, a time dependent colon drug delivery system can be used.

Naturally, the formulations according to the invention may also contain one or more additives conventionally used in mucosal drug delivery, such as preservatives, stabilizers, inert excipients, etc. Agents suitable for these and other purposes are known to anyone skilled in the art of mucosal drug delivery.

Preparation of formulations

The chitosan and therapeutic active agent containing formulation may be prepared in a buffered saline solution at a neutral pH, i.e. pH 6.5-7.5. A lower pH may be used for easier dissolvation of the chitosan by addition of HCl to the afore-mentioned buffer or a different buffer system. The person skilled in the art will be able to determine the optimal pH which may be between 1.0 and 11.0 and preferably between 4.0 and 7.5.

The chitosan and therapeutic agent formulation may be prepared in any forms suitable for mucosal delivery, such as oral or rectal delivery, such as tablets (e.g. immediate release tablets, enteric coated tablets, extended release tablets), gelatine capsules, suppositories, microspheres, gels, solutions, suspensions, etc.

The invention will now be illustrated but not limited by the following examples.

Example 1: Toxicity of chitosans as assessed with a dehydrogenase activity assay in Caco-2 cells

Preparation of chitosans with different molecular weight and degree of acetylation

The preparation and characterization of chitosans was performed according to procedures described in Anthonsen et al., Carbohydrate Polymers, 22, 1993, pp 193-201. The chitosans used in the example are given in table 1.

Table 1: Chitosan samples used for structure-effect studies.

Chitosan	Degree of acetylation (%)	Molecular weight
1	1	31000
2	1	170000
3	35	5300
4	35	12000
5	35	170000
6	49	22000

7	49	98000
8	15	4700
9	15	190000

Preparation of chitosan solutions

Chitosan solutions were prepared by dissolving them at concentration of 0.5 and 1.0 mg/ml in Hank's balanced salt solution buffered with 25 mM 2N morpholino ethane sulfonic acid to a pH=5.5 (HBSS pH=5.5). This pH value is comparable to the pH of the small intestine microclimate and facilitates the dissolutions of the chitosans. HBSS is a buffer commonly used in cell culture experiments. Phosphate buffered saline (PBS) and similar physiological buffers could also be used as solvents. Chitosans were dissolved by vigorously shaking for 24 to 48 hours. Just prior to experiments the chitosan solutions were diluted to the appropriate concentrations with HBSS pH=5.5.

Dehydrogenase activity assay

In these studies Caco-2 cells obtained from American Type Culture Collection, Rockville, USA, were used. Cells of passage number 93-105 were used throughout. The cells were seeded in 96-well tissue culture plates (Flow Laboratories, Irvine, UK). After 24 h they were exposed to different concentrations of chitosans for 60 min at 37 C in air. Subsequently, the intracellular dehydrogenase activity was determined by the MTT method as described in Anderberg et al., Pharm. Sci., 81, 1992, 879-887. MTT is a tetrazolium salt that is cleaved by mitochondrial dehydrogenases in living but not dead cells to give a dark blue product. Following addition of MTT, the cells were incubated for another 60 min. The developed colour was measured with a multiwell scanning spectro photometer. The

dehydrogenase activity was evaluated as the relative absorbance at 590 nm of the solutions in the 96-well plate as compared to control, the latter being 100%.

Figure 1 shows the effect of chitosans on intracellular dehydrogenase activity. The data are given as the mean of 3 experiments. In general, the difference between individual data points is less than 25%. In figure 1 the different curves relate to (■) chitosan (DA=1%, Mw=31000), (●) chitosan (DA=1%, Mw=170000); (◊) chitosan (DA=15%, Mw=4700); (▼) chitosan (DA=15%, Mw=190000); (▲) chitosan (DA=35%, Mw=12000); (♦) chitosan (DA=35%, Mw=170000); (□) chitosan (DA=49%, Mw=22000); (o) chitosan (DA=49%, Mw=98000); (▵) chitosan (DA=35%, Mw=5300). For chitosans with a low degree of acetylation chitosan (DA=1%, Mw=31000); chitosan (DA=1%, Mw=170000); chitosan (DA=15%, Mw=4700); and chitosan (DA=15%, Mw=190000); a dose dependent effect on intracellular dehydrogenase activity is observed. Chitosans with an intermediate degree of acetylation and large molecular weight (chitosan (DA=35%, Mw=170000)) decrease enzyme activity only at higher concentrations. Chitosan (DA=35%, Mw=5300), chitosan (DA=35%, Mw=12000), chitosan (DA=49%, Mw=22000), and chitosan (DA=49%, Mw= 98000) do not show an effect in the concentration range studied.

Example 2: Toxicity of chitosans as assessed by effects on epithelial cell morphology

Caco-2 cells were obtained from American Type Culture Collection, Rockville, USA. The cells were cultivated on polycarbonate filters (Transwell cell culture inserts, Costar, Cambridge) as described by Artursson, Pharm. Sci., 79, 1990, 476-482. Cells of passage 93-105

were used throughout. The cells were used for experiments 21 to 35 days after seeding. After washing the monolayers apically with HBSS pH=5.5 and basolaterally with Hank's balanced salt solution buffered with 25 mM Hepes to pH=7.4 (HBSS pH=7.4), chitosans were added apically, while the basolateral solution was replaced with fresh HBSS pH=7.4. The chitosans described in example 1 were diluted from stock solutions as described in example 1. All chitosans were added at a concentration 50 µg/ml, except chitosan (DA=49%, Mw=98000) which was used at 250 µg/ml. Following exposure to the different chitosan solutions for 60 min at 37°C in air, the cell monolayers were rinsed twice with PBS and fixed in 1.5% glutaraldehyde and 1% osmium tetroxide, and immersed in 1% uranyl acetate. Thin sections were examined with a Philips 420 electron microscope at 60 kV.

In monolayers treated with 50 µg/ml chitosan (DA=1%, Mw=31000) and chitosan (DA=1%, Mw=170000) discontinuities in and reduced number of microvilli were observed, while the terminal web showed a disorganized pattern. The tight junctions appeared not to be effected. Following treatment with 50 µg/ml chitosan (DA=35%, Mw=170000), and 250 µg/ml chitosan (DA=49%, Mw=98000) the structural changes were not as pronounced. Microvilli and terminal web appeared normal. See figure 2 which is a transmission electron micrograph of a) control cells (magnification 1950x), b) cells treated for 60 min with 50 µg/ml chitosan (DA=1%, Mw=31 000)(magnification 2500x) and c) cells treated for 60 min with 50 µg/ml chitosan (DA=35%, Mw=170 000) (magnification 2500x). Exposure to chitosan (DA=35%, Mw=5300); chitosan (DA=35%, Mw=12000); chitosan (DA=49%, Mw=22000) did not result in obvious structural changes.

Example 3: Absorption enhancement of chitosans - effect on epithelial permeability

Caco-2 cell monolayers were used as a model for mucosal and in particular intestinal epithelium. Drug transport across Caco-2 cell monolayers has in the past been demonstrated to correlate excellently with in vivo human intestinal drug absorption. In the presence of absorption enhancer, correlations between in vitro cell culture experiments and in vivo drug absorption have also been established, with corrections in the applied dose due to upscaling sometimes being necessary. Mannitol was used as a model compound for hydrophilic drugs which show a low and incomplete uptake across mucosal epithelium. Transport of mannitol in Caco-2 cell monolayers has previously been shown to correlate with the transport of sparingly absorbed drugs within a molecular weight range of 100-500. All transport experiments were performed in HBSS. Prior to the experiments the cells were washed apically with HBSS pH=5.5, and basolaterally with HBSS pH=7.4. Just before the experiments chitosans described in example 1 were diluted from stock solutions with HBSS pH=5.5 to the appropriate concentrations as described in example 1. ^{14}C-mannitol was subsequently added. The experiments were initiated by replacing the apical blank HBSS pH=5.5 medium for a ^{14}C-mannitol/chitosan solution, and the basolateral solution for fresh HBSS pH=7.4. Transport studies were performed during 120 min in air at 37 °C and 95% relative humidity. Samples were taken at regular time intervals from the basolateral side, and were counted in a liquid scintillation counter (Tricarb, 1900CA). The apparent permeability (Papp) was calculated using the following equation:

$Papp = dQ/dt \times 1/(AxCo)$, in which dQ/dt is the rate of appearance of mannitol on the basolateral side, Co is the initial mannitol concentration on the apical side,

and A is the surface area of the monolayer.

The mannitol permeability of the Caco-2 cell monolayers could be improved by some but not all of the chitosans studied. Table 2 shows the mean permeability over 120 min during exposure to 50 µg/ml chitosan solution. The effect of the chitosans was dependent on both the degree of acetylation and the molecular weight of the polymer. At a concentration of 50 µg/ml, chitosans with a low degree of acetylation showed a clear effect on mannitol transport. Chitosan (DA=1%, Mw=31000); chitosan (DA=1%, Mw=170000); chitosan (DA=15%, Mw=4700); chitosan (DA=15%, Mw=190000); showed a mean Papp between 0 and 120 min that was increased 5.5- to 16.1-fold, as compared to control. Chitosans with a larger degree of acetylation and a low or intermediate molecular weight, such as chitosan (DA=35%, Mw=5300), chitosan (DA=35%, Mw=12000) and chitosan (DA=49%, Mw=22000) did not influence mannitol transport significantly at concentrations of 50 µg/ml. Chitosan, however, with a high molecular weight and intermediate degree of acetylation (DA=35%, Mw=170000) strongly increased the Papp of mannitol. Chitosan with a very large degree of acetylation, but also a high molecular weight (DA=49%, Mw=98000), showed a small but significant increase in mannitol transport at 50 µg/ml.

Table 2: Effect of different chitosans at a concentration of 50 µg/ml on the permeability of mannitol across Caco-2 cell monolayers.

Chitosan (µg/ml)	Degree of acetylation (%)	Molecular weight	Papp x 10E7 (cm/sec)	Ratio Papp chitosan /Papp control
control		-	2.2 ± 0.2	1
50	1	31000	28.9 ± 5.8	13.2
50	1	170000	24.0 ± 0.7	10.9
50	35	5300	2.3 ± 0.8	1.1
50	35	12000	2.7 ± 0.2	1.3
50	35	170000	29.0 ± 3.8	13.2
50	49	22000	4.8 ± 4.4	2.2
50	49	98000	9.0 ± 1.4	4.1
50	15	4700	12.8 ± 3.4	
50	15	190000	35.5 ± 10.4	

The mean Papp for ^{14}C-mannitol are calculated over the total 120 min. period of the experiments. The Papp values are given as the mean ± s.d. of 3-4 experiments.

Example 4: Absorption enhancement of chitosans - concentration dependency

Mannitol transport experiments were performed with different concentrations of chitosans to study the concentration dependency of the absorption enhancing effect. The chitosans used in the experiments are described in example 1. Solutions having a concentration of 10, 50 and 250 µg/ml were prepared according to example 1. The transport experiments were similarly performed as in example 3.

The chitosan effect on mannitol transport was concentration dependent. The effect of chitosans at 3 different concentrations 10, 50, and 250 µg/ml on the Papp of mannitol across Caco-2 cell monolayers are shown in Figure 3. The Papp is calculated as the mean Papp during the 120 min time period of the experiment. The data are given as the mean ± s.d. of 3-4 experiments. (■) chitosan (DA=1%, Mw=31000); (●) chitosan (DA=1%, Mw=170000); (◊) chitosan (DA=15%, Mw=4700); (▼) chitosan (DA=15%, Mw=190000); (▲) chitosan (DA=35%, Mw=12000); (♦) chitosan (DA=35%, Mw=170000); (□) chitosan (DA=49%, Mw=22000); (○) chitosan (DA=49%, Mw=98000); (△) chitosan (DA=35%, Mw=5300).

A 10 µg/ml dose gave a small increase in mannitol transport for chitosan (DA=1%, Mw=31000), chitosan (DA=1%, Mw=170000), and chitosan (DA=15%, Mw=190000) while all other chitosans did not have an effect at this concentration. At 250 µg/ml chitosan (DA=1%, Mw=31000), chitosan (DA=1%, Mw=170000), chitosan (DA=15%, Mw=4700) and chitosan (DA=15%, Mw=190000) increased the permeability to about 50 to 60 times the control value. For chitosan (DA=35%, Mw=170000) the mean Papp at 250 µg/ml was not significantly different from the Papp value obtained with 50 µg/ml. Chitosan (DA=49, Mw=98000) increased the Papp approximately 10 times at 250 µg/ml.

Example 5: Absorption enhancement of chitosans - onset of action of increased epithelial permeability

Similar transport experiments as described in example 3 were done to study the onset of action of increased permeability of chitosans. The chitosans used in the experiments and the preparation of the different

solutions are described in example 1.

The onset of action of chitosans that increased epithelial permeability was not the same for chitosans. Although the mean Papp measured over 120 min was similar for chitosan (DA=1%, Mw=31000), chitosan (DA=1%, Mw=170000), chitosan (DA=15%, Mw=190000) at a concentration of 50 µg/ml, and chitosan (DA=49%, Mw=98000) at a concentration of 250 µg/ml, the permeabilities at 20 min after the start of the experiment were very different. Papp values at 20 min are summarized in table 3. Exposure to chitosan with intermediate degree of acetylation and high molecular weight (DA=35, Mw=170000) showed a much stronger increase in permeability after 20 min than all other chitosans (compare figure 3 and table 2 with table 3).

Table 3: Effect of different chitosans on the permeability of mannitol across Caco-2 cell monolayers after 20 min exposure.

Chitosan (µg/ml)	Degree of acetylation (%)	Molecular weight	Papp x 10E7 (cm/sec)	Ratio Papp chitosan /Papp control
control		-	2.7 ± 0.5	1
50	1	31000	4.4 ± 0.6	1.6
50	1	170000	3.9 ± 0.4	1.4
50	35	5300	3.3 ± 0.8	1.2
50	35	12000	2.9 ± 0.2	1.1
50	35	170000	8.4 ± 1.8	3.1
50	49	22000	1.7 ± 0.3	0.6
250	49	98000	3.8 ± 0.7	1.4
50	15	4700	4.3 ± 1.0	
50	15	190000	5.0 ± 0.4	

The Papp for ^{14}C-mannitol are calculated over the first 20 min period of the experiments. The Papp values are given as the mean ± s.d. of 3-4 experiments.

Example 6: Absorption enhancement of chitosans - effect on the epithelial permeability of atenolol

Just before the start of the experiments chitosan (DA=1%, Mw=31.000) and chitosan (DA=35, Mw=170.000), as described in example 1, were diluted from stock solutions with HBSS pH=5.5 to a concentration of 50 µg/ml, as described in example 1. Caco-2 cell transport

experiments were performed, subsequently, as described in example 3. Atenolol concentrations were determined using HPLC consisting of a RP Ultrasphere ODS 5 µm (4.6 mm x 25 cm) column connected to a UV detector. Absorption was measured at 270 nm. The mobile phase consisted of phosphate buffer (pH=3) and acetonitrile in a ratio of 90:10.

Figure 4 shows the Papp of atenolol during exposure to 50 µg/ml chitosan solution. The data are given as mean ± SD of 4 experiments. In figure 4 the different curves relate to (x) control; (■) chitosan (DA=1%, Mw=31000); and (♦) chitosan (DA=35, Mw=170000). The permeability of the Caco-2 cell monolayers was low ($0.5\text{-}1.0\times10^{-7}$). The permeability could be improved, however, by chitosan (DA=1%, Mw=31.000) and chitosan (DA=35, Mw=170.000). Similarly, as observed for mannitol permeability the effect of chitosan (DA=35, Mw=170.000) was larger than the effect of chitosan (DA=1%, Mw=31.000) during the early stages of the experiment. For chitosan (DA=35, Mw=170.000) the atenolol permeability did not increase further after 20 min exposure, while for chitosan (DA=1%, Mw=31.000) atenolol permeability kept increasing until the end of the experiment.

CLAIMS

1. A pharmaceutical formulation c h a r a c-
t e r i z e d in that it comprises a chitosan polymer having a degree of acetylation of between 20 and 45% and a molecular weight of above 75,000, and a therapeutic active agent.

2. A pharmaceutical formulation according to claim 1 c h a r a c t e r i z e d in that the degree of acetylation of the chitosan polymer is between 30 and 40%

3. A pharmaceutical formulation according to claim 1 c h a r a c t e r i z e d in that the molecular weight of the chitosan polymer is between 75,000 and 250,000, and preferably between 98,000 and 200,000.

4. A pharmaceutical formulation according to claim 1 c h a r a c t e r i z e d in that the therapeutic active agent is selected from the group drugs, vaccines, proteins, peptides and fragments thereof.

5. A chitosan polymer for preparing the formulation according to claim 1 c h a r a c t e r i z e d in that it is a chitosan polymer having a degree of acetylation of between 20 and 45% and a molecular weight of above 75,000.

6. A chitosan polymer according to claim 5 c h a r a c-
t e r i z e d in that the degree of acetylation of between 30 and 40%.

7. A chitosan polymer according to claim 5 c h a r a c-
t e r i z e d in that the molecular weight is between 75,000 and 250,000, and preferably between 98,000 and

200,000.

8. Use of a chitosan polymer according to claim 5 in the manufacture of a pharmaceutical formulation comprising a chitosan polymer having a degree of acetylation of between 20 and 45% and a molecular weight of above 75,000 and a therapeutic active agent.

9. A method for enhancing the absorption of a therapeutic active agent by administering a sufficient amount of a formulation comprising a chitosan polymer having a degree of acetylation of between 20 and 45% and a molecular weight of above 75,000 and said therapeutic active agent to a host in need of such a therapeutic treatment.

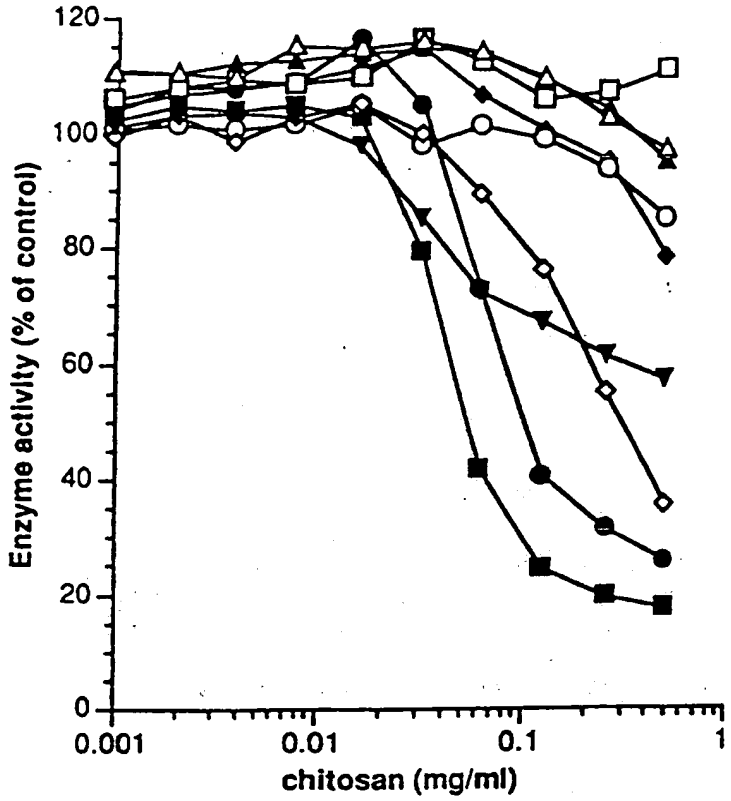
120
100
80
60
40
20
0
Enzyme activity (% of control)
0.001
0.01
0.1
1
chitosan (mg/ml)

Figure 1

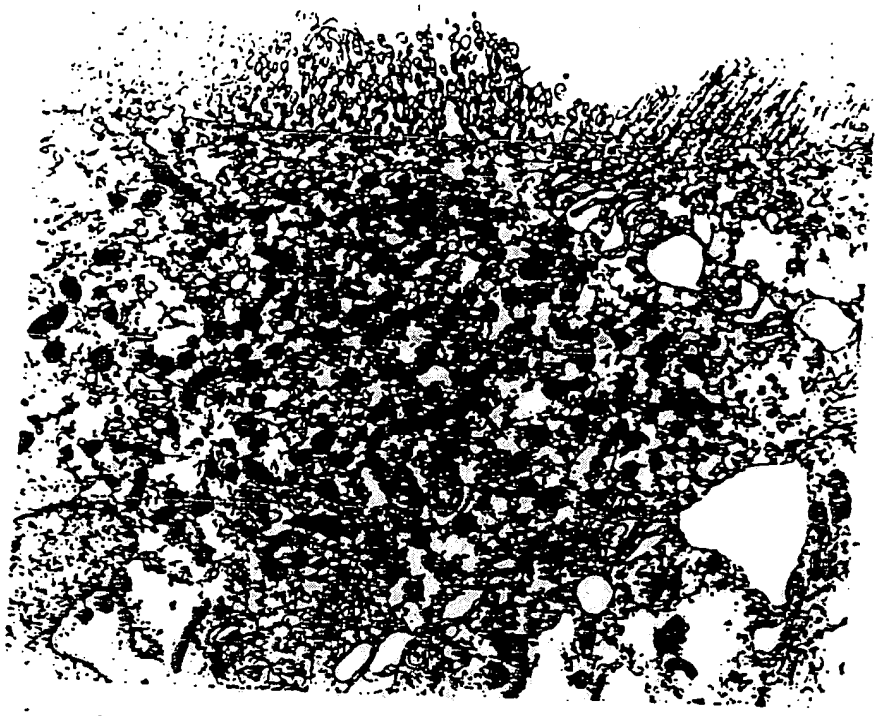

Fig. 2a

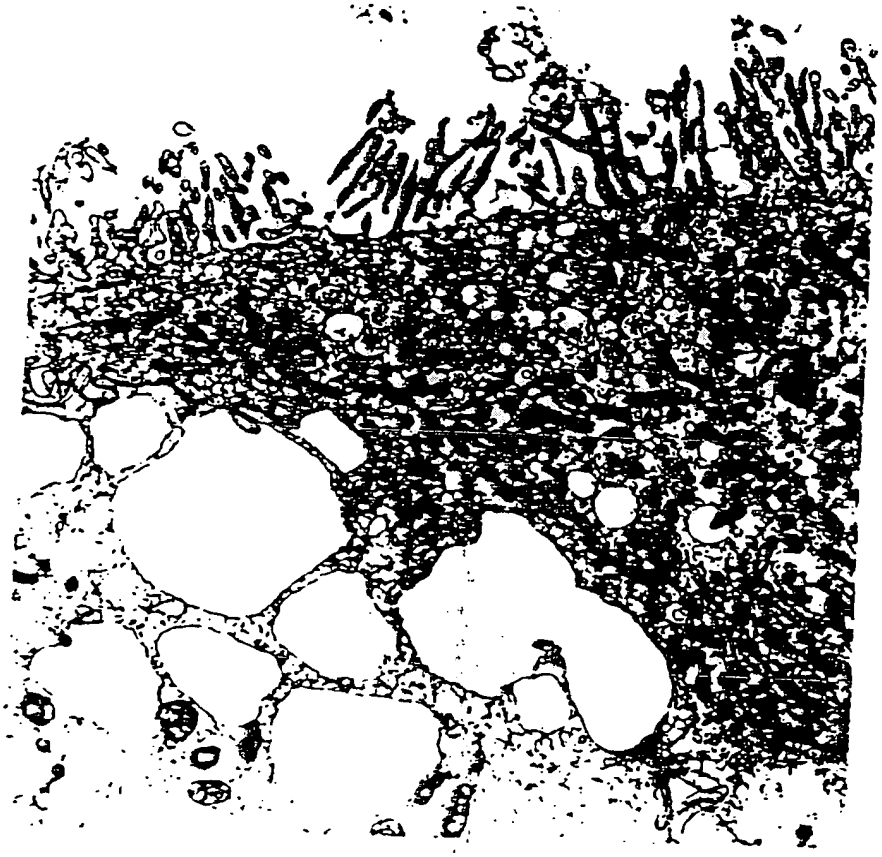

Fig. 2b

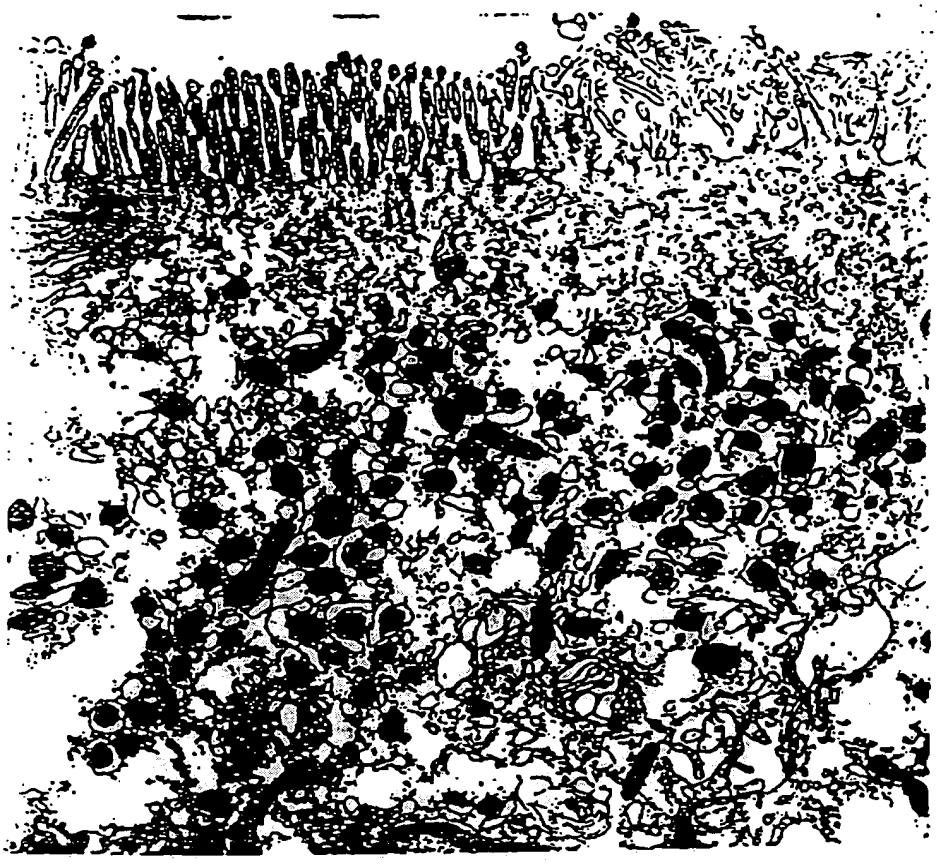

Fig. 2c

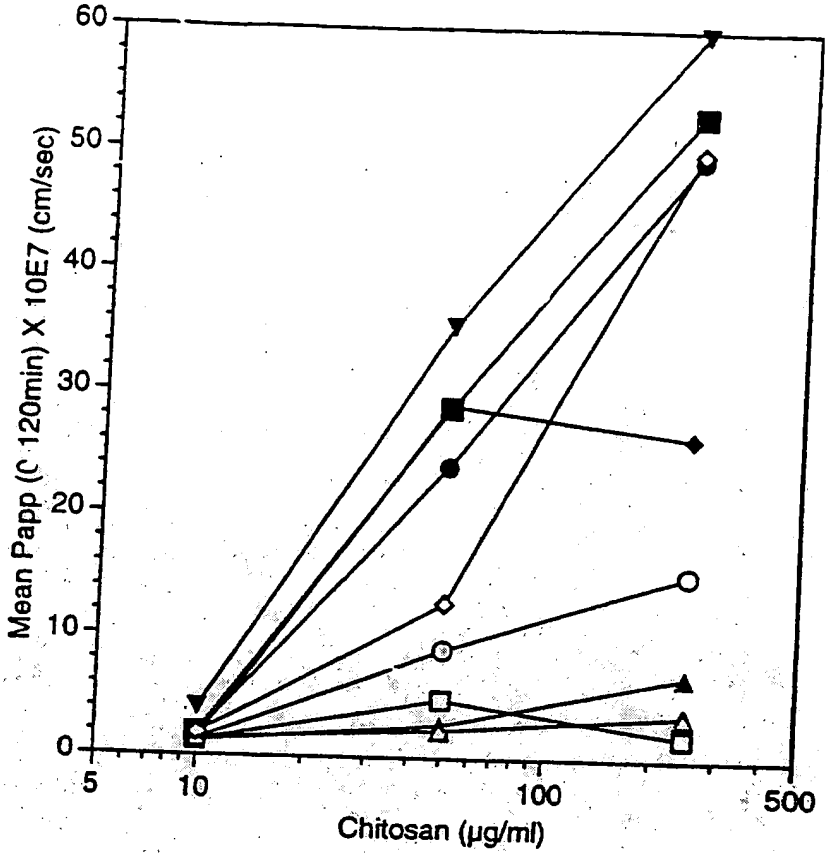
Mean Papp (C 120min) X 10E7 (cm/sec)
60
50
40
30
20
10
0
5
10
100
500
Chitosan (µg/ml)

Figure 3

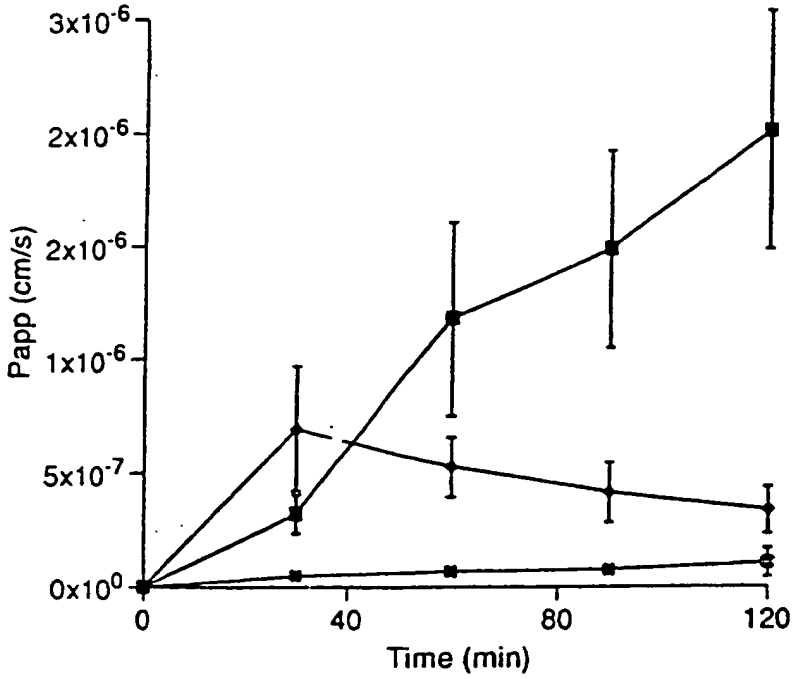
3x10-6
2x10-6
2x10-6
1x10-6
5x10-7
0x100
Papp (cm/s)
0
40
80
120
Time (min)

Figure 4

INTERNATIONAL SEARCH REPORT

International application No.
PCT/SE 95/01342

A. CLASSIFICATION OF SUBJECT MATTER

IPC6: A61K 47/36, C08L 5/08
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC6: A61K, C08L

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

SE,DK,FI,NO classes as above

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

MEDLINE, EMBASE, WPI, WPIL, CLAIMS, CA

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	US 4873092 A (ICHIRO AZUMA ET AL), 10 October 1989 (10.10.89)	1-9
X	INTERNATIONAL JOURNAL OF PHARMACEUTICS, Volume 67, 1991, Teruko Imai et al, "Interaction of indomethacin with low molecular weight chitosan, and improvements of some pharmaceutical properties of indomethacin by low molecular weight chitosans" page 11 - page 20	1-9
A	WO 9009780 A1 (DANBIOSYST UK LIMITED), 7 Sept 1990 (07.09.90)	1-9

[X] Further documents are listed in the continuation of Box C. [X] See patent family annex.

* Special categories of cited documents:
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"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"X" document of particular relevance: the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"Y" document of particular relevance: the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
27 February 1996	29.02.96
Name and mailing address of the ISA/ Swedish Patent Office Box 5055, S-102 42 STOCKHOLM Facsimile No. +46 8 666 02 86	Authorized officer Anneli Jönsson Telephone No. +46 8 782 25 00

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INTERNATIONAL SEARCH REPORT

International application No.
PCT/SE 95/01342

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	Dialog Information Services, File 73, EMBASE, Dialog accession no. 9214951, EMBASE accession no. 94156779, Luessen H.L. et al: "Bioadhesive polymers for the peroral delivery of peptide drugs", J. CONTROL. RELEASE (Netherlands), 1994, 29/3 (329-338)	1-9

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INTERNATIONAL SEARCH REPORT

International application No.
PCT/SE 95/01342

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
US-A- 4873092	10/10/89	NONE	
WO-A1- 9009780	07/09/90	DE-D,T- 69008346	25/08/94
		EP-A,B- 0460020	11/12/91
		SE-T3- 0460020	
		ES-T- 2055904	01/09/94

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